# DIFFERENTIAL GENE EXPRESSION OF ANTIOXIDANT ENZYMES IN MACROPHAGES EXPOSED TO CARBON PARTICLES ADSORBED WITH BENZO-[a]-PYRENE.

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## Introduction

Airborne particulate matter can carry pollutants to the deep distal lung bypassing the respiratory defenses. In urban environments, up to 50% of the respirable particulate matter is carbonaceous. The major source of carbonaceous particles are cigarette smoke, diesel exhaust and incomplete combustion of fossil fuels (1-2). After deposition on bronchial and alveolar epithelium, the load of carbonaceous particles, if not exceeding the normal clearance capacity, are removed by the resident macrophages. Macrophages containing carbonaceous particles have been found in sputum of smokers (1). It has been hypothesized that the impairment of macrophage clearance of carbonaceous particles and the pollutants that they carry, mainly the polyaromatic hydrocarbons, may contribute to the lung-associated injury, presumably initiated by the inflammatory responses that may result in the release of cytokines inducing inflammatory cell recruitment, epithelial cell hypertrophy and hyperplasia (3). If the exposure is chronic, the persistence of these cellular functions can eventually lead to tumorigenesis. Unlike the other environmental pollutants such as ozone, sulfur dioxide and nitrogen dioxide, little is known about the mechanism of particle-induced lung injury. Since most pollutants produce injury that is oxidant-mediated and that cells respond to oxidant-stress by increasing the expression of antioxidant enzymes and stress response genes (4), we investigated whether exposure to carbonaceous particles induce expression of antioxidant enzymes and stress-response genes in macrophages and if so, how are they regulated. We have focused our studies on the stress-response gene product called heme oxygenase 1 (HO-1).

HO-1 is a microsomal membrane enzyme that catalyzes the first and rate-limiting reaction in heme catabolism, to yield equimolar quantities of biliverdin, iron and carbon monoxide. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. There are two isoforms of heme oxygenase, HO-1, the inducible form and HO-2, the constitutive form. Exposure of mammalian cells to cellular stresses such as heme, hypoxia, hyperoxia, lipopolysaccharide, cytokines, heavy metals, ultraviolet irradiation, gluthathione depletors, and hyperthermia have been shown to induce HO-1 gene expression (5-8). A common feature among the various inducers of HO-1 is that these agents, including heme, generate production of reactive oxygen species and/or modify gluthathione levels. This correlation and the observation that bilirubin, one of the end products of heme catabolism, functions as an antioxidant, has led to the hypothesis that HO-1 induction is part of a general response to oxidant stress and that this enzyme plays a protective role during such conditions (9).

In this study we hypothesized that carbon particles carrying the pollutant, Benzo-[a]-pyrene induce HO-1 gene expression in macrophages.We further examined the molecular regulation of HO-1 induction by carbon particles in macrophages.

## Materials and Methods

*Cell culture* - A rat peritoneal macrophage cell line RAW 264.7 were obtained from American Tissue Cell Culture and were maintained in Dulbecco's Modified Eagles Medium supplemented with 10 % fetal bovine serum and gentamicin (50 µg/ml). Cultures were maintained at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. All experiments were performed with confluent cultures.

*Model Carbon Particles* -Two model carbon particles N339 and N339ox had preadsorbed on their surfaces a 0.75 monolayer of Benzo-[a]-Pyrene. To prevent agglomeration, the model particles were homogenized in DMEM media at 3000 *rpm* for 1 hour. Cells were exposed to N339+BAP and N339ox+BAP at [2 µg/ml] for up to 24 hours. Controls used for this study were model particles alone (N339 and N339ox) and also BAP alone. When stated, cells were pretreated with Actinomycin D (0.5 µg/ml), Cyclohexamide (1 µg/ml), Cytochalasin B (10 µg/ml) and N-Acetyl-cysteine (20 mM) for 1 h prior to carbon exposure.

*RNA Extraction and Northern Blot Analysis* - Total RNA was isolated by the STAT-60 RNAzol method with direct lysis of cells in RNAzol lysis buffer followed by chloroform extraction (10). Northern Blot analysis were performed as described

previously (11). Briefly, 10 µg aliquots of total RNA were fractionated on a 1% denaturing agarose gel, transferred to a nylon membrane by capillary action and cross-linked to the membrane by UV irradiation. The nylon membranes were incubated in hybridization buffer (1% bovine serum albumin, 7% SDS, 0.5M phosphate buffer, pH 7.0, 1.0 mM EDTA) containing $^{32}P$ labelled rat HO-1 cDNA (12) at 65°C for 24 hours. Nylon membranes were then washed twice in buffer A (0.5% bovine serum albumin, 5% SDS, 40 mM Phosphate buffer, pH 7.0, 1.0 mM EDTA) for 30 min. at 55°C followed by 4 washes in buffer B (1%SDS, 40mM Phosphate buffer pH 7.0, 1.0 mM EDTA) for 15 minutes at 55°C and exposed to X-OMAT film. To normalize for the amount of RNA in different samples or loading errors, blots were stripped and hybridized with radiolabelled oligonucleotide probe (5'-ACGGTATCTGATCGATCGTCTTCGAACC-3') complimentary to 18S rRNA. The values for the HO-1 mRNA transcript (1.8kb) were normalized to values for 18s rRNA obtained on the same blot. The HO-1 mRNA levels in the RAW exposed to model particles were expressed in densitometric absorbance units, normalized to control untreated samples, and expressed as fold induction relative to controls.

*Western Blot Analyses* - Cells were homogenized in lysis buffer (1% Nonidet P-40, 20 mM Tris, pH 8.0, 137.5 mM NaCl, 1 mM $Na_2VO_4$, 1 mM phenylmethylsulfonylfluoride, 10 µg aprotinin). Protein concentration of the lysates were determined by coomasie blue dye-binding assay (BioRad, NJ). An equal volume of 2X sample buffer (0.125M Tris-Hcl, pH 7.4, 4% SDS and 20% glycerol) was added to the sample and boiled for 5 minutes. Samples (100 µg) were electrophoresed in a 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membranes were incubated with a rabbit polyclonal antibody against HO-1 (1:1000 dilution) for 1.5 hours following incubation with goat anti-rabbit IgG antibody for 1.5 hours. Signal development was carried out using an ECL detection kit (Amersham Corp, England).

*DNA fragmentation assay* - Genomic DNA were extracted as directed by the manufacturer for the Puregene™ DNA isolation kit (Gentra, NC). Twenty µg of DNA was load onto a 1.5% agarose gel in 1X Tris-Acetate buffer and subjected to electrophoresis.

## RESULTS

*Exposure to model particles adsorbed with Benzo-[a]-Pyrene (N339+BAP and N339ox+BAP) induces HO-1 gene expression in RAW cells* -Cells were exposed to 2 µg/ml of both model particles, N339+BAP and N339ox+BAP for 1 h, 2 h, 4 h, 8 h and 24 h and HO-1 gene expression was examined by Northern blot analyses. There was a marked increase in the steady-state levels of HO-1 mRNA was observed at 4 h with the highest level of induction obtained after 8h of continuous exposure to N339+BAP and N339ox+BAP (Figure 1a). The accumulation of HO-1 mRNA levels correlated with increased HO-1 protein levels (Figure 1b).

*Induction of HO-1 expression in RAW cells is dependent on phagocytosis of the model particles.*
Cells exposed to N339+BAP for 8h demonstrated a 22 fold increase in HO-1 mRNA (Figure 2).To demonstrate that internalization of N339+BAP is required for HO-1 induction, we pretreated the RAW cells with 10 µg/ml of cytochalasin B, a potent inhibitor of phagocytosis for 1 h prior to exposure. Cytochalasin B pretreatment inhibited the N339+BAP induced HO-1 mRNA accumulation. Cytochalasin B treatment alone did not affect HO-1 mRNA levels.

*HO-1 mRNA is dependent on gene transcription and de novo protein synthesis.*
To further delineate the molecular basis for increased expression of HO-1 in response to N339+BAP, we examined whether HO-1 mRNA induction was dependent on gene transcription. Cells were pretreatment for 1 h with Actinomycin D, a potent inhibitor of RNA synthesis, prior to an 8 h exposure to N339+BAP. As shown in Figure 3, actinomycin D completely prevented N339+BAP induced HO-1 mRNA accumulation. We then determined whether HO-1 mRNA induction is dependent on *de novo* protein synthesis. Cells were pretreated with cycloheximide, a potent inhibitor of protein synthesis, for 1 h prior to an 8 h exposure to N339+BAP. Cyclohexamide also completely inhibited N339+BAP induced HO-1 mRNA expression.

*Do reactive oxygen species (ROS) mediate N339+BAP induced HO-1 expression?*
We determined whether phagocytosis of the model particle N339+BAP liberate ROS that may be responsible for inducing HO-1 expression. Pretreatment of cells with an antioxidant N-acetyl-cysteine (20 mM) 1 h prior to exposure to N339+BAP did not prevent the increase of HO-1 mRNA (Figure 4).

*Phagocytosis of the model particle +BAP induces apoptosis in macrophages*
Cells exposed to both model particles, N339+BAP and N339ox+BAP, for 24 h underwent apoptosis. Characteristic endonuclease generated DNA fragments of 160-180 bp are evident in RAW cells after 24h of continuous model particle exposure (Fig. 5)

## Discussion

There has been much interest generated recently by reports demonstrating the induction of HO-1 gene expression by a variety of pro-oxidants (ultraviolet irradiation, hyperoxia and lipopolysaccharide) (13-16). This study demonstrates that exposure to model particles preadsorbed with Benzo-[a]-pyrene (N339+BAP and N339ox+BAP) also induces HO-1 gene expression in macrophages. The model particles selected for this study are well-defined carbon particles which are carbon blacks manufactured under conditions determined by the American Society for Testing Material (17). Since the surfaces of the environmental carbonaceous particles are heterogeneous, containing both oxidized and non-oxidized active sites, we decided to separate them and study their potential adverse effects. As controls for this study, the model particles alone (N339 and N339ox) as well as BAP separately did not induce HO-1 gene expression significantly (data not shown). The criteria for HO-1 induction requires both types of model particles to be a) adsorbed with BAP and b) internalized. The observation that we do not have a significant attenuation of HO-1 gene expression after pretreatment of the cells with NAC may suggest that HO-1 induction may not be modulated by reactive oxygen species. We have also examined other "prototypical" antioxidant enzymes that were exposed to these model particles. There were no significant changes in the mRNA levels of both CuZnSOD and MnSOD (data not shown).

Programmed cell death or apoptosis is a gene regulated process in which coordinated series of morphological changes such as nucleus and chromatin condensation, cell membrane blebing and fragmentation of cell into membrane-bound apoptotic bodies occur resulting in cell death. Removal of apoptotic bodies by phagocytosis by neighboring cells, in particular macrophages, occurs without initiating inflammation. Apoptosis is often a physiologic process, especially important during embryogenesis, organ atrophy and normal adult tissue turnover. However, accumulating evidence suggest that genotoxic and oxidant stress can induce cell death via apoptosis. Preliminary studies show that carbon particles can induce apoptosis in macrophages, and further studies are necessary to understand the regulation and function of carbon-induced apoptosis.

Future work will focus on delineating the transcriptional regulation and signal transduction pathways involved in the activation of the HO-1 gene by carbon particles. Furthermore, we will investigate whether carbon-induced HO-1 expression can serve to protect the macrophages from further cellular oxidant stress.

## References


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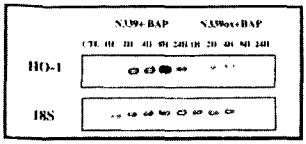


Fig. 1a. Northern blot analyses of HO-1 in RAW cells. Total RNA was extracted at the indicated times following continuous exposure to the model particles and analyzed for HO-1 mRNA expression. The ribosomal RNA 18s is shown as a normalization control.

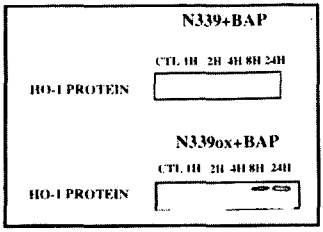


Fig. 1b. Western blot analyses of HO-1 protein in RAW cells. Total protein was extracted at the times indicated following continuous exposure to model particles and probed for HO-1 protein.

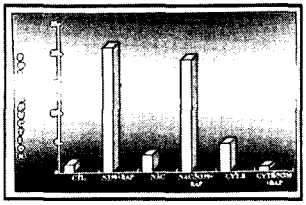

Fig. 2. Effects of NAC (20mM) and cytochalasin B (4ug/ml) on RAW cells exposed continuously to 8h N339+BAP. NAC pretreatment prior to N339+BAP exposure had no effect on HO-1 mRNA expression in RAW cells exposed to N339+BAP. Cytochalasin B pretreatment prior to exposure inhibited HO-1 mRNA expression. The results represent mean fold inducton by Northern blot analyses. 18s rRNA hybridization was used as a normalization control.

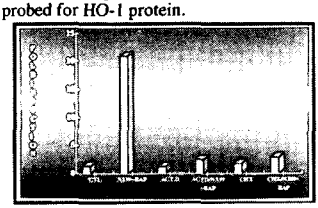

Fig. 3. Effects of Act. D (0.5ug/ml) and cyclohexamide (1ug/ml) on RAW cells exposed continuously to 8h N339+BAP. Act. D pretreatment prior to N339+BAP exposure prevented HO-1 mRNA expression in RAW cells exposed to N339+BAP. Cyclohexamide pretreatment prior to exposure also inhibited HO-1 mRNAexpression. The results represent mean fold induction by Northern blot analyses. 18s rRNA hybridization was used as a normalization control.

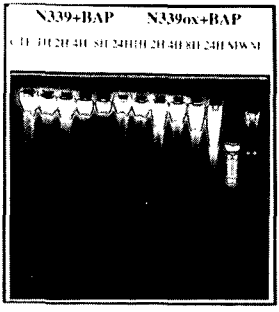


Fig. 4. Genomic DNA fragmentation gel assay of RAW cells. DNA (20 ug) was extracted at the times indicated following continuous exposure to model particles. Characteristic DNA fragments of 160-300 base pairs are evident at 24h of model particle exposure.